Polycyclic Aromatic Compounds, 32:141–153, 2012
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ISSN: 1040-6638 print / 1563-5333 online
DOI: 10.1080/10406638.2011.640733

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Gene Expression Changes of Phases I and II Metabolizing Enzymes Induced by PAH Derivatives

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, and generate various types of PAH derivatives, such as nitrated PAHs, hydroxylated PAHs, and PAH quinones through chemical reactions in the atmosphere. PAHs are well known to activate the aryl hydrocarbon receptor (AhR), followed by the induction of metabolizing enzymes mainly in the liver, while biological responses to PAH derivatives are not understood well. In this study, we investigated the induction patterns of gene expression of CYP1 family and Phase II metabolizing enzymes in rat H4IIE cells exposed to PAH quinones and their parent PAHs for 24 h.

Dibenz[*a,h*]anthracene and benzo[*a*]pyrene dramatically induced mRNA expression of CYP1 family, such as cytochrome P450 (*Cyp*) *1a1*, *Cyp1a2*, and *Cyp1b1*, and PAH quinones, especially 1,4-chrysenequinone, possess a high potential to induce CYP1 family. As for Phase II enzymes, PAHs induced NAD(P)H: quinone oxidoreductase 1 (*Nqo1*) and UDP-glucuronosyltransferase (*Ugt*)*1a6*, and their induction potencies by PAHs were similar to those of CYP1 family. On the other hand, expression of sulfotransferase (*Sult*)*1a1*, heme oxygenase-1 (*Hmox1*), and *Ugt2b1* were augmented mainly by PAH derivatives. Finally, we examined gene expression changes of metabolizing enzymes by the airborne particles. Their organic extracts significantly up-regulated the

Received 14 September 2011; accepted 10 November 2011.
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expression of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Ugt1a6*, and *Nqo1*, but not *Hmox1*, *Ugt2b1*, and *Sult1a1*.

These results suggest that PAHs mainly induce the expression of genes encoding CYP1 family while PAH derivatives, especially quinones, induce the expression of genes encoding both CYP1 family and Phase II enzymes. Furthermore, our results show the organic chemicals which adsorb on the airborne particles exert biological effects in the similar manner of PAHs, suggesting the involvement of mainly AhR activation.

Key Words: AhR, metabolizing enzyme, PAH, PAH derivatives

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), which generated from the combustion of fossil fuels and biomass burnings, cause various health effects through the diverse changes of gene expression and signal transduction. In the atmosphere, PAHs generate their derivatives, such as nitro PAHs (NPAHs) (1), hydroxylated PAHs (OHPAHs), and PAH quinones (PAHQs) through the chemical reactions of nitrogen radicals ($\cdot NO^3$), hydroxide radicals ($\cdot OH$), and ultraviolet light (2).

Many studies have shown that PAHs and PAH derivatives have various physiologic actions *in vivo* and *in vitro*. For instance, PAHs activate the aryl hydrocarbon receptor (AhR), which is a transcriptional factor and play a pivotal role in the PAH toxicity (3). On the other hand, the derivatization of PAHs gives PAHs different functions from those of the PAHs. It has been reported that PAHQs have toxicities related to oxidative stress-producing reactive oxygen species (ROS) (4). OHPAHs have estrogenic/antiestrogenic activity, suggesting that they act as endocrine disruptors (5). In most cases, these biological effects of PAHs and PAH derivatives are caused concomitantly with gene expression changes, since PAHs and PAH derivatives activate various transcriptional factors. It is anticipated that such gene expression changes may be useful for understanding the biological effects of PAHs and their derivatives, and may also be useful as biomarkers.

When PAHs and PAH derivatives enter into animal bodies, they induce the expression of genes encoding metabolizing enzymes, such as CYP1 family (e.g., cytochrome P450) and Phase II enzymes (e.g., (NAD(P)H: quinone oxidoreductase 1 and heme oxygenase-1), via transcriptional regulation in the liver (6–8). Since PAHs and PAH derivatives have different physiological effects, it is speculated that numerous kinds of atmospheric pollutants show various regulation of genes, such as metabolizing enzymes. However, it has not been fully understood how the derivatization of PAHs changes the physiological effects under gene expression level.

Although derivatization of 4-rings PAH, especially quinones, gives biological effects such as ROS generation (4) and estrogenic/antiestrogenic activities (5), there are few studies about comparison of cytochromes P-450 between

parent PAHs and their derivatives (9). In this study, we investigated the induction potency of genes encoding CYP1 family and Phase II metabolizing enzymes by PAHs and PAH derivatives having 4-rings. In addition, we examined gene expression changes by 5-rings PAHs, which have strong activities of AhR activation, as a substance strongly inducing the genes of CYP1 family. Four-rings and 5-rings PAHs and their derivatives are the main components adsorbed in airborne particles, and are thought to be involved in most of toxicities caused by airborne particles. Therefore, in order to explore mechanisms under which PAHs and their derivatives adsorbed in airborne particles cause biological effects, we also examined the expression changes of CYP1 family and Phase II genes by exposure to the extracts of airborne particles.

MATERIALS AND METHODS

Chemicals

We used four PAHs and four PAH derivatives (Figure 1), which show AhR activation by dioxin-responsive chemical-activated luciferase gene expression (DR-CALUX) assay according to the previous report (10,11). 3,4-Dihydrobenz[*a*]anthracen-1(2H)-one (3,4-OH-B[*a*]A) was purchased from Sigma-Aldrich Corp. (Tokyo, Japan). Dibenz[*a, h*]anthracene (DBA) was

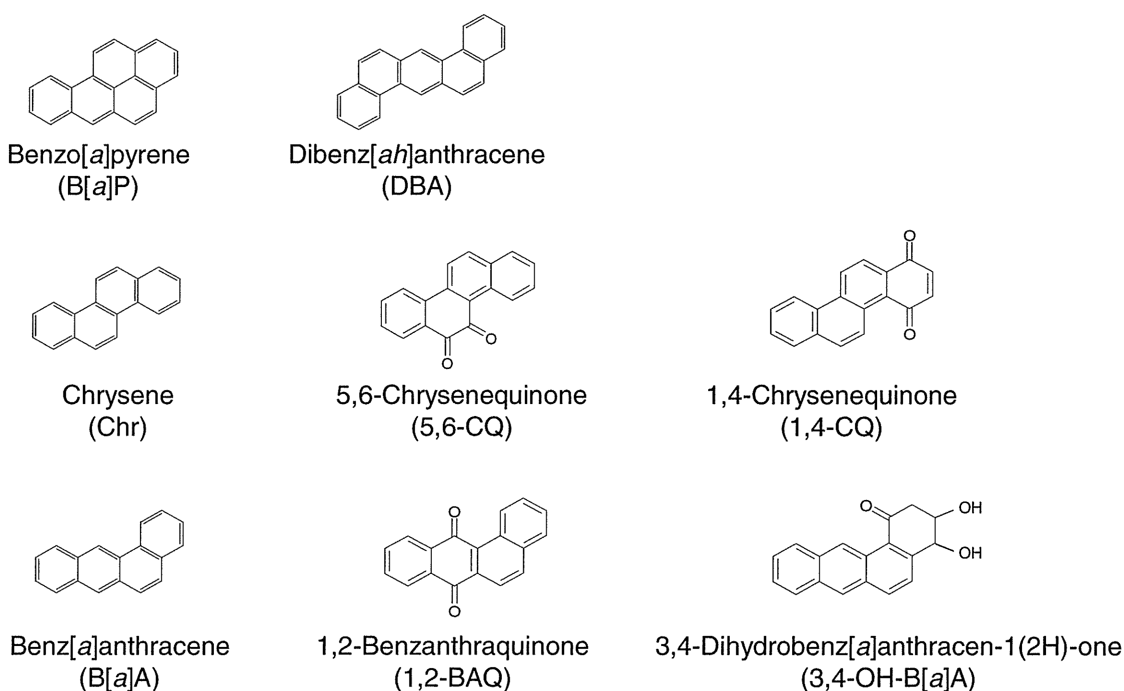

Figure 1: Structures of PAHs and PAH derivatives tested in this study PAH: B(a)P, DBA, Chr, B(a)A, PAH derivatives: 5,6-CQ, 1,4-CQ, 1,2-BAQ, 3,4-OH-B(a)A.

purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). 5,6-Chrysenequinone (5,6-CQ) and 1,4-chrysenequinone (1,4-CQ) were purchased from Chiron AS (Trondheim, Norway). Benz[*a*]anthracene (B[*a*]A), chrysene (Chr), benz[*a*]pyrene (B[*a*]P) and 1,2-benzanthraquinone (1,2-BAQ) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). These chemicals were dissolved in dimethyl sulfoxide (DMSO).

Airborne Particle Extracts

Airborne particles were collected on the roof of the 5th floor building of the research center for eco-environmental science from the Chinese Academy of Science in Beijing, China for 10 days in March and April in 2008 using high-volume air samplers (Kimoto Electric Company Limited, Osaka, Japan) at a flow rate of 700 l/min on the quartz fiber filters (2500QAT-UP, Pallflex Products) (Putnam, CT, USA). Every 24 h, the filter was changed. The amount of volume of air that passed through each filter was 16,800 m^3. After being dried in the desiccator in the dark, the filters were weighed and then stored in a refrigerator (−20°C) until use. The average weight of airborne particles of each filter was 4.8 g and the particle concentration was calculated to be approximately 300 $\mu g/m^3$ on average. Extraction was conducted from these filters with benzene/ethanol (3:1, v/v), and then the extract solution was filtered by ADVANTEC filter paper (125 mm in diameter) (Toyo Roshi, Tokyo, Japan). The extracts were concentrated by evaporation and residue was dissolved in DMSO. Among ten day samples we chose three which show relativity high AhR agonist activity by DR-CALUX.

H4IIE Cell Culture and RNA Isolation

H4IIE cell line rat liver cancer cells were obtained from BioDetection Systems B.V. (Amsterdam, The Netherlands). The cells were cultured in alpha-minimal essential medium (α-MEM) (Invitrogen Corp., NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. The cells were seeded into a 6-well plate, and grown up to 75% confluence. Then, the cells were exposed to each PAH and PAH derivative in triplicate in a final concentration of 4 μM. These concentration was in the range from 1–10 μM which didi not show any cytotoxicities in our previous study (10). DMSO alone (0.4%) was used as a control group. In the experiment of exposure to the airborne particles, cells were exposed to the extracts of airborne particles (corresponding to 3.2 mg particles/well). These concentrations showed no cytotoxicity to the cells in our previous study (10) and thus selected as testing concentraions. After 24 h exposure, the medium was removed, and then total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Valencia). The total RNA concentration was determined spectrometrically, and quickly frozen at −80°C until use.

Quantification of mRNA Encoding Metabolizing Enzymes by Real Time PCR

First-strand cDNA of each gene was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Tokyo, Japan). 1 μg of total RNA was added to 2 μl random hexamer primer (600 pmol/μl), and then the mixture was heated at 65°C for 10 min. Four μl of transcriptor RT reaction buffer, 0.5 μl of protector RNase inhibitor (40 U/μl), 2 μl of deoxynucleotide mix (10 mM each) and 0.5 μl of transcriptor reverse transcriptase were added to the reaction mixture, and they were heated at 55°C for 30 min, followed by reaction at 85 °C for 5 min to inactivate the enzyme. Expression of genes encoding CYP1 family (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*), Phase II (*Nqo1*, *Ugt1a6*, *Ugt2b1*, *Sult1a1,* and *Hmox1*) metabolizing enzymes and β-actin (housekeeping) were evaluated using TaqMan master (Roche, Tokyo, Japan) and a LightCycler® 480 system (Roche Applied Science). Primers and hybridization probes were designed using the LightCycler® Probe Design Software 2.0. Oligonucleotides used for RT-PCR were commercially synthesized by Japan Gene Research Laboratories Inc. (Sendai, Japan) (Table 1).

The relative changes in gene expression were calculated by the ddCt method (12.13) for the mRNA expression of CYP1 family and Phase II metabolizing enzymes, using the following formula: $\mathrm{ddCt} = [C_{t\text{-gene}} - C_{t\text{-}\beta\text{-actin}}]_{\mathrm{Sample}} - [C_{t\text{-gene}} - C_{t\text{-}\beta\text{-actin}}]_{\mathrm{Control}}$. The expression was normalized with β-actin, and the relative gene expression was calculated by the equation $2^{-\mathrm{ddCt}}$.

Table 1: List of primers used for quantitative RT-PCR

Primer	Sequence (5′-3′)
Phase I	
Cyp1a1	gagaaagatccaggaggagttaga
	gggacaaaggatgaatgtcg
Cyp1a2	tcctacaactctgccagtctcc
	cctctcaacacccagaacact
Cyp1b1	ctgctctacaccgctggaa
	tcagctgctgtggactgtct
Phase II	
Nqo1	agcgcttgacactacgatcc
	caatcagggctcttctcacc
Ugt1a6	aagcgatggaaattgctgag
	cgatggtctagttccggtgt
Ugt2b1	tttggtttcaaccatttaaagaga
	gccttccccatcatctcag
Sult1a1	acacatctgcccctgtcct
	gcatttcgggcaatgtaga
Hmox1	gtcaggtgtccagggaagg
	ctcttccagggccgtataga

RESULTS

Expression Changes of Genes Encoding CYP1 Family Metabolizing Enzymes in PAH and PAH Derivative-exposed Cells

We investigated expression changes of *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* genes, which encode cytochrome P450 1a1, 1a2, and 1b1, respectively, when cells were exposed to each PAH and PAH derivative (Figure 2). Because B[*a*]P and DBA are well known as strong AhR agonists (14), we used them to show gene expression changes elicited by pathways through AhR activation. Predictably, both B[*a*]P and DBA induced the expression of *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* (Figure 2A). Four-rings PAHs, Chr and B[*a*]A, significantly induced the expression of *Cyp1a1*, although their effects were less than those of B[*a*]P and DBA (Figure 2B). In addition, Chr and B[*a*]A induced the same expression level of *Cyp1a2* and *Cyp1b1*, while B[*a*]P and DBA exerted a greater effect on the expression of *Cyp1b1* than that of *Cyp1a2* (Figure 2A). On the other hand, quinone derivatives of Chr and B[*a*]A also induced the expression of *Cyp1a1*, *Cyp1a2* and *Cyp1b1* (Figure 2B). 1,2-BAQ significantly induced the genes of CYP1 family, and was comparable in degree to the parent PAH, while 3,4-OH-B[*a*]A showed no ability to induce the genes of CYP1 family. Surprisingly, 1,4-CQ more strongly induced the expression of genes of CYP1 family than its parent PAH (Chr), although 5,6-CQ more weakly induced the expression of genes of CYP1 family than Chr.

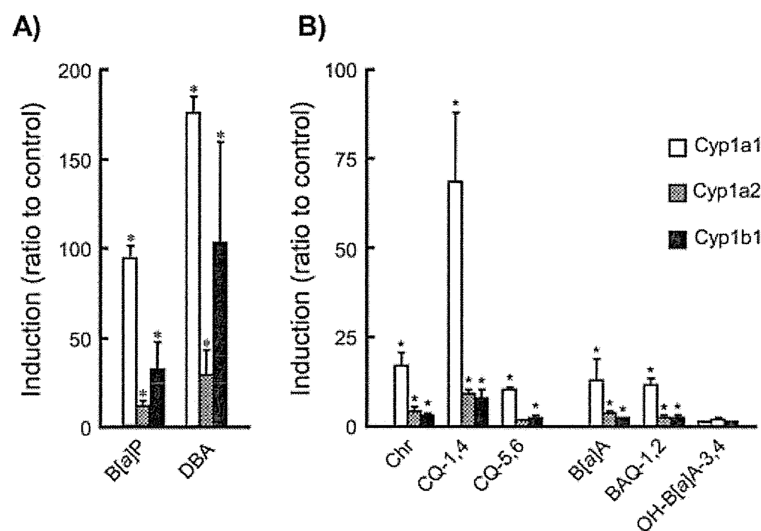

Figure 2: mRNA expression of CYP1 family metabolizing enzymes by PAHs and PAH derivatives. The cells were exposed to PAHs and PAH derivatives at concentration of 4 μM for 24 h. Symbols and vertical bars respectively represent the mean and ±S. D. ($n = 3$). Asterisk (*) shows significant difference ($p < 0.05$).

Expression Changes of Genes Encoding Phase II Metabolizing Enzymes in PAH and PAH Derivative-exposed Cells

We examined changes of gene expression of Phase II metabolizing enzymes, *Ugt1a6*, *Nqo1*, *Hmox1*, *Ugt2b1*, and *Sult1a1*, which encode UDP-glucuronosyltransferase (Ugt) 1a6 and 2b1, NAD(P)H dehydrogenase quinone 1, heme oxygenase-1, and sulfotransferase 1a1, respectively. The expression of *Ugt1a6* and *Nqo1* were significantly induced by some of the PAHs or PAH derivatives (Figure 3A), which relatively strongly induced the genes of CYP1 family. On the other hand, the expression of *Hmox1*, *Ugt2b1*, and *Sult1a1* showed no or weak inductions by the compounds examined in this study (Figure 3B). Exceptionally, expression of *Ugt2b1* was up-regulated solely

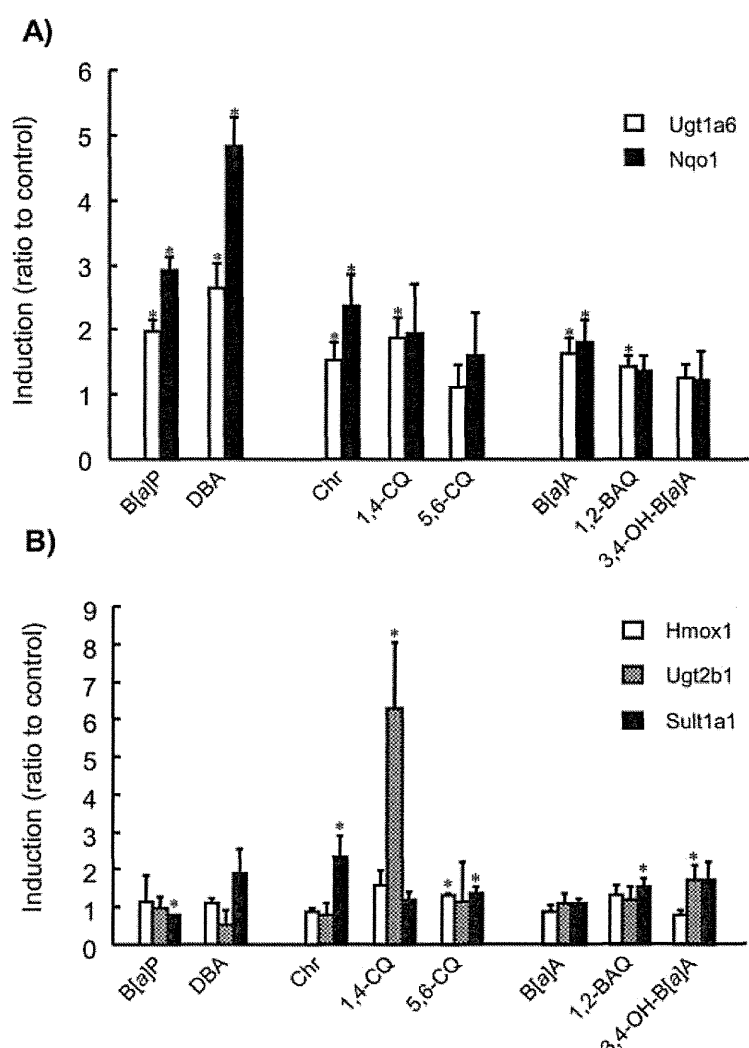

Figure 3: mRNA expression of Phase II metabolizing enzymes by PAHs and PAH derivatives. (A) Ugt1a6, Nqo1; (B) Hmox1, Ugt2b1, Sult1a1. The cells were exposed to PAHs and PAH derivatives at concentration of 4 μM for 24 h. Symbols and vertical bars respectively represent the mean and ±S. D. ($n = 3$). Asterisk (*) shows significant difference ($p < 0.05$).

by exposure to 1,4-CQ, although Chr and 5,6-CQ did not up-regulate the expression of this gene.

Expression Changes of Genes Encoding CYP1 Family and Phase II Metabolizing Enzymes by the Extracts of Airborne Particles

The extracts of airborne particles strongly induced the expression of *Cyp1a1* (Figure 4), and mildly that of *Cyp1a2* and *Cyp1b1*. In addition, they slightly, but significantly, up-regulated the expression of *Ugt1a6* and *Nqo1*, but not that of *Hmox1*, *Ugt2b1*, and *Sult1a1*. There were no large differences between each sample of airborne particles.

DISCUSSION

Liver metabolizes xenobiotic substances entering into the body, leading to the formation of numerous kinds of PAH derivatives. Not only PAHs but also their derivatives formed from this metabolization pathway induce CYP1 family and Phase II metabolizing enzymes. Since PAHs and PAH derivatives are

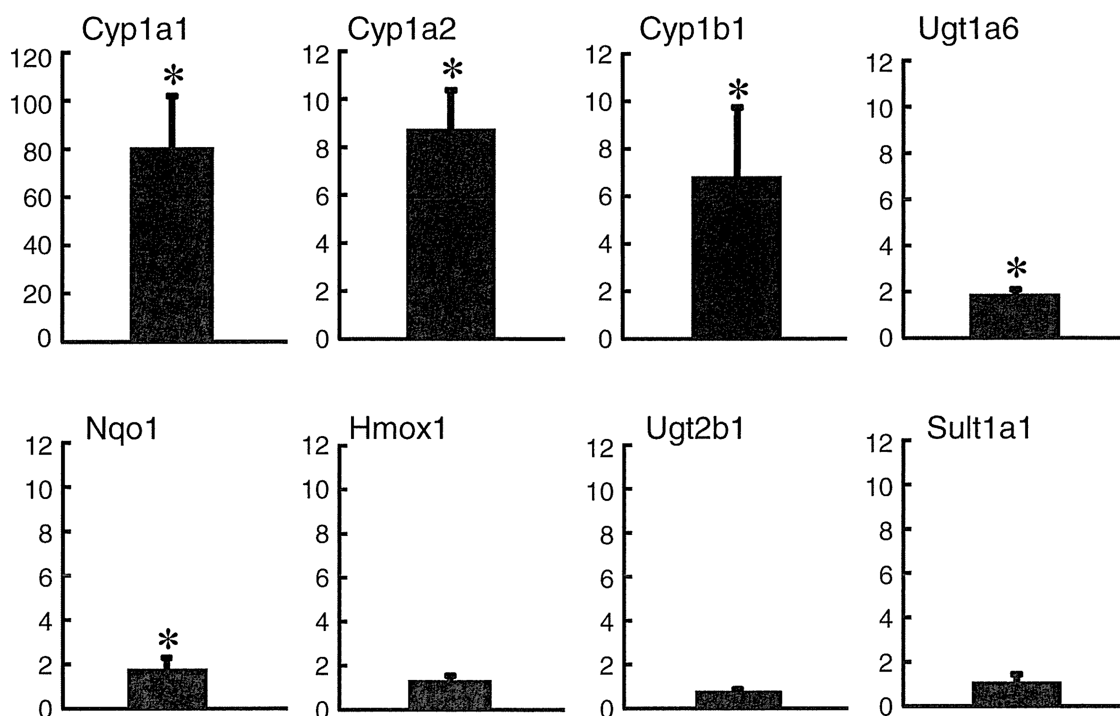

Figure 4: mRNA expression of CYP1 family and Phase II metabolizing enzymes by airborne particles. The cells were exposed to airborne particles for 24 h. Symbols and vertical bars respectively represent the mean and ±S. D. ($n = 3$). Asterisk (*) shows significant difference ($p < 0.05$).

distributed among various organs in the body, this metabolism is linked to toxicity in many cases. Therefore, toxicological analysis using liver cells is a useful method for toxicological evaluation of xenobiotic substances.

We showed here PAHs- and PAH derivatives-induced changes of expression levels of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Ugt1a6*, *Nqo1*, *Hmox1*, *Ugt2b1*, and *Sult1a1*. CYP1 family is well known as the Phase I enzyme, and play a pivotal role. Ugt family facilitates detoxification of the carcinogen of derivatives formed by CYP1 family (15).

In this study, in order to know the induction of genes encoding the metabolizing enzymes through AhR activation, we used two 5-rings PAHs, B[*a*]P and DBA, which tremendously induced the expression of *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* (Figure 2A), 5′-flanking regions of which have several AhR binding sites, termed xenobiotic responsive element (XRE). B[*a*]P and DBA also upregulated the levels of *Ugt1a6* and *Nqo1* (Figure 3A). *Ugt1a6* and *Nqo1* also have the XRE in their enhancer regions, and are regulated through binding of AhR/ARNT complex to this *cis*-element (15,16). These results were consistent with the previous reports (17). On the other hand, *Hmox1*, *Ugt2b1*, and *Sult1a1* were not induced by exposing to these two strong AhR agonists (Figure 3B), indicating that *Hmox1*, *Ugt2b1*, and *Sult1a1* are not regulated directly and indirectly by the AhR. It has been reported that expression of *Hmox1*, *Ugt2b1*, and *Sult1a1* is regulated by several transcriptional factors other than AhR, such as the activator protein 1 (AP-1) (18), androgen receptor (AR) (19), constitutive androstane receptor/constitutive active receptor (CAR) (20), glucocorticoid receptor (GR) (19), and nuclear factor erythroid 2-related factor 2 (Nrf2) (20). In addition, our results show that B[*a*]P significantly inhibited the expression of *Sult1a1*. This result is supported by the previous study showing that 3-methyochoranthrene, a strong AhR agonist, decreases the expression of *Sult1a1* (21), although its mechanism remains unclear.

In order to study the effects of derivatization on gene expression changes in CYP1 family and Phase II enzymes, 4-rings PAHs, Chr and B[*a*]A, were examined in this study. These two PAHs induced the expression of CYP1 family at a relatively low level, compared with 5-rings PAHs, suggesting that 4-rings PAHs have weaker potency for AhR activation than 5-rings PAHs. Quinone derivatization of B[*a*]A did not induce extreme changes of gene expression of CYP1 family elicited by B[*a*]A. We also found that 3,4-OH-B[*a*]A did not change the expression of CYP1 family, *Ugt1a6* and *Nqo1*, while it has a tendency to increase the expression of a part of Phase II genes that are not regulated by the AhR, suggesting a possibility that the increasing polarity of PAHs leads to the shift of inductive pattern of metabolizing enzymes.

Interestingly, 1,4-CQ more strongly induced the expression of these CYP1 family than the parent compound (Chr), while 5,6-CQ that is a structural isomer of 1,4-CQ inversely show lower induction of CYP1 family than Chr. This tendency was almost in parallel with AhR binding activities of the compounds

by DR-CALUX (10). Our data suggest that there are cases where metabolism of some PAHs rather enhance the induction of CYP1 family.

The expression levels of *Cyp1a2* and *Cyp1b1*, which are also known as the AhR target genes, were up-regulated by exposure to PAHs and PAH derivatives examined in the present study (Figure 2). Our data show that B[*a*]P and DBA increased the expression of *Cyp1b1* stronger than that of *Cyp1a2*, while B[*a*]A, Chr and 1,4-CQ mildly induced the same levels of expression of *Cyp1a2* and *Cyp1b1*. This discrepancy of induction pattern of two genes among PAHs may be due to the difference of transcriptional regulation between *Cyp1a2* and *Cyp1b1*. Although both genes are induced by the AhR agonist, XRE motif has not identified in the up-stream region of *Cyp1a2*, and AhR/ARNT heterodimer is not able to bind to this region. Sogawa et al. reported a novel function of AhR/ARNT as a coactivator (22). In addition, enhancer region of *Cyp1a2* has AP1 binding site (23). On the other hand, steroidgenic factor-1 (SF-1) motif, cAMP responsive element (CRE), and E-Box were found in 5′ flanking region of *Cyp1b1* (24,25). Therefore, future study is needed to examine whether there is difference of activation of transcriptional factors involved in the regulation of genes of CYP1 family other than AhR between 4- and 5-rings PAHs.

Uniquely, the expression of *Ugt2b1* was increased remarkably only by 1,4-CQ, although this mechanism remains unclear. As shown in Figure 2, 1,4-CQ strongly induced the expression of *Cyp1a1*, suggesting that this compound activates the AhR. In addition, PAHQ produces ROS (4), resulting in activation of the transcriptional factors related to the oxidative stress, such as Nrf2, which regulates the expression of Ugt2b1(20). However, other than *Ugt2b1*, Nrf2 has been reported to regulate the expression of *Hmox1*(26), which was not significantly induced by 1,4-CQ. Therefore, regulation of *Ugt2b1* by 1,4-CQ may be involved in unidentified other transcriptional factor(s).

Finally, we analyzed the induction potency of genes encoding CYP1 family and Phase II metabolizing enzymes by the extracts from airborne particles collected in Beijing, China. The airborne samples strongly induced up-regulation of *Cyp1a1*, and mild up-regulation of *Cyp1a2* and *Cyp1b1* in the liver cells (Figure 4). In addition, the extracts significantly up-regulated the expression of *Ugt1a6* and *Nqo1*, but not *Hmox1*, *Ugt2b1*, and *Sult1a1*. Since these pattern of gene expression changes are similar to those by B[*a*]P and DBA, it seems that the effects of the extracts at the transcriptional levels of CYP1 family and Phase II genes are mainly attributed to the AhR activation. In addition, the induction potency of *Cyp1b1* by the extracts from the airborne particles was almost same level with that of *Cyp1a2*. This result was similar to those by 4-rings PAHs and their derivatives. However, the extracts did not up-regulate the expression of Phase II enzymes that were regulated by 4-rings PAHs and their derivatives, and gene expression changes of metabolizing enzymes

between exposure to single compound and crude extracts were slightly different. The airborne particles used in this study were collected from China, because this sample contains very much higher concentration of various PAHs than that from other locations, such as England, Greece, and Japan (27–29). Therefore, the similarity between gene expression patterns of airborne particles in China and some PAHs obtained in this report may be due to the relatively high concentration of PAHs.

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Polycyclic Aromatic Compounds, 32:177–187, 2012
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ISSN: 1040-6638 print / 1563-5333 online
DOI: 10.1080/10406638.2011.654307

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Atmospheric Formation of Hydroxynitrofluoranthene from Photochemical Reactions of 2-Nitrofluoranthene

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The formation of hydroxynitrofluoranthene (OHNF) via photochemical reactions of 2-nitrofluoranthene (2-NF) was demonstrated using a UV irradiation system. The photoreaction of 2-NF in acetonitrile/water (3/1, v/v) gave a product that was hydroxynitro-substituted. Moreover, we detected an OHNF isomer, which was found in the 2-NF photoreaction products, in soluble organic fractions of ambient airborne particles collected in Kanazawa, Japan. The atmospheric concentration of the OHNF isomer was estimated to be less than 2 fmol m^{-3}, which was comparable to that of 1-hydroxy-3-nitropyrene, but lower than that of 1-hydroxy-6-nitropyrene by a factor of 10. The results suggests that atmospheric OHNF is partly formed *via* secondary formation processes, i.e., photochemical reactions of 2-NF appear to have a significant effect on the occurrence of OHNF in the atmosphere.

Key Words: 2-nitrofluoranthene, airborne particles, atmospheric secondary formation, hydroxynitrofluoranthene, photochemical reaction

INTRODUCTION

Nitrated polycyclic aromatic hydrocarbons (NPAHs) are a class of mutagens/carcinogens found in the atmosphere, and some of them exhibit stronger

Received 31 October 2011; accepted 30 December 2011.
This research was supported by MEXT/JSPS Grant-in-Aid for Scientific Research (21200031, 22510010) and the Environment Research and Technology Development Fund (RF-0905) of the Ministry of the Environment, Japan.
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mutagenicity/carcinogenicity than their parent polycyclic aromatic hydrocarbons (PAHs) (1). Some types of NPAH are formed via gas-phase reactions of semi-volatile PAHs. For example, 2-nitropyrene (2-NP) is formed from the gas-phase reaction of pyrene with OH radicals in the presence of NO_2, and 2-nitrofluoranthene (2-NF), one of the most abundant NPAHs in the atmosphere, is formed via two pathways, i.e., OH or NO_3 radical-initiated reactions in the gas phase (2). On the contrary, 1-nitropyrene (1-NP) is a representative NPAH formed through combustion of fossil fuels such as diesel fuel. Nitropyrenes and nitrofluoranthenes taken up by humans and animals are transformed to various metabolites such as hydroxynitropyrenes (OHNPs) and hydroxynitrofluoranthenes (OHNFs) in the presence of cytochrome P450 enzymes (3, 4). Several isomers of OHNP, such as 1-hydroxy-3-nitropyrene (1-OH-3-NP), 1-hydroxy-6-nitropyrene (1-OH-6-NP), and 1-hydroxy-8-nitropyrene (1-OH-8-NP) have also been observed in airborne particles (5, 6) and diesel exhaust particles (DEP) (7–9). Several studies have found that most OHNP and OHNF isomers have lower mutagenic activity than the parent NPAHs (9–11). Recently, however, we have found that several OHNPs act as endocrine disruptors, i.e., they act as estrogenic, anti-estrogenic, and anti-androgenic compounds (12, 13), which may cause dysfunction of human and wildlife endocrine systems, abnormal development of reproductive systems, and immunodeficiencies. In view of the influence of hydroxynitro-aromatics on human health, we need to learn more about their environmental concentrations, sources and behavior.

Photoreactions of 1-NP have been studied both in solvents (14–23) and on solid substrates (15, 19, 24–26). The products of these reactions include OHNPs such as 1-hydroxy-2-nitropyrene (1-OH-2-NP) (14), 1-OH-3-NP, 1-hydroxy-5-nitropyrene (1-OH-5-NP), 1-OH-6-NP, 1-OH-8-NP (6), 2-hydroxy-1-nitropyrene (2-OH-1-NP) (18), and 9-hydroxy-1-nitropyrene (9-OH-1-NP) (17). However, nothing is known about the photoreaction products of 2-NF, which is the most abundant NPAH in the atmosphere. Although several OHNP isomers have been observed in ambient samples (5, 6), there is not yet any evidence that OHNFs, which may be formed from the photoreaction of 2-NF, exist in the atmosphere. Therefore, in this study, we examined the formation of OHNF from photochemical reactions of 2-NF by laboratory experiments in order to clarify whether OHNF occurs in the atmosphere. Furthermore, we report that a particle-associated OHNF that was found in the laboratory photoreaction products was also detected in the atmosphere.

EXPERIMENTAL SECTION

Experimental Setup for Photoreaction of 2-NF

Photoreaction of 2-NF was performed in a Pyrex sleeve (1 cm in thickness) which surrounded an annular Pyrex vessel (6.6 cm ID × 60 cm length) as

previously reported (6). The external sleeve has a port for sampling the photoreaction products and the precursor 2-NF. The radiation equipment has 6 black-light lamps (20 W, Toshiba, FL20S-BLB) and a cooling device for isothermal reaction conditions at 299 ± 2 K. The 1×10^{-6} mol L^{-1} of 2-NF in acetonitrile/water (3/1, v/v) in the external sleeve was irradiated by the black light lamps placed around the reaction vessel under the presence of air. The total incident photon flux reaching to the surface of the sleeve measured with a Hatchard-Parker actinometer using potassium ferrioxalate photoreduction was 5.2×10^{16} photons cm^{-2} s^{-1}. The maximum intensity of the photon flux was obtained at a wavelength of 350 nm. A product collected after 2 h of the reaction was analyzed by high-performance liquid chromatograph equipped with a chemiluminescence detector (HPLC/CL) or liquid chromatographic-tandem mass spectrometer (LC/MS/MS) after acid-base and preparative HPLC fractionations.

Airborne Particle Collection

Airborne particles were collected at the rooftop level of a 7-story building approximately 30 m above ground level at Kanazawa University, Kanazawa, Ishikawa, Japan. This sampling site is located in a typical suburban area. The sampling campaign was performed using a high-volume air sampler (Kimoto Electric, Model 120) on quartz fiber filters (QFF; Advantec MFS, QR100), at a flow rate of 1500 L min^{-1}, on November 12, 2007 for 6 h. The airborne particle samples were stored at 253 K until subjected to analysis.

Extraction of Soluble Organic Fractions (SOF) from Airborne Particulate Samples

The filter samples were cut into fine pieces before extraction. SOF from the filter samples were extracted twice with 100 mL of dichloromethane under sonication for 20 min. The extract solution was filtered with a cellulose acetate filter to remove solid residue, followed by concentrating to ca. 5 mL by a rotary evaporator. The sample solution after acid-base and preparative HPLC fractionations was subjected to the quantification by HPLC/CL.

Sample Fractionation

In order to analyze the OHNF in 2-NF photoreaction products and the SOF from airborne particles, sample fractionation was performed by the following procedure. The crude SOF extracted from airborne particles or obtained from the photoreaction of 2-NF were separated by acid-base partitioning according to the previous reports (6, 28) in order to remove basic and neutral fractions containing interfering compounds such as parent 2-NF (Figure 1). The obtained weak-acidic fraction (WA_P) with phenolic compounds such as OHNFs was applied to further fractionation by preparative HPLC (Hitachi, L-6200)

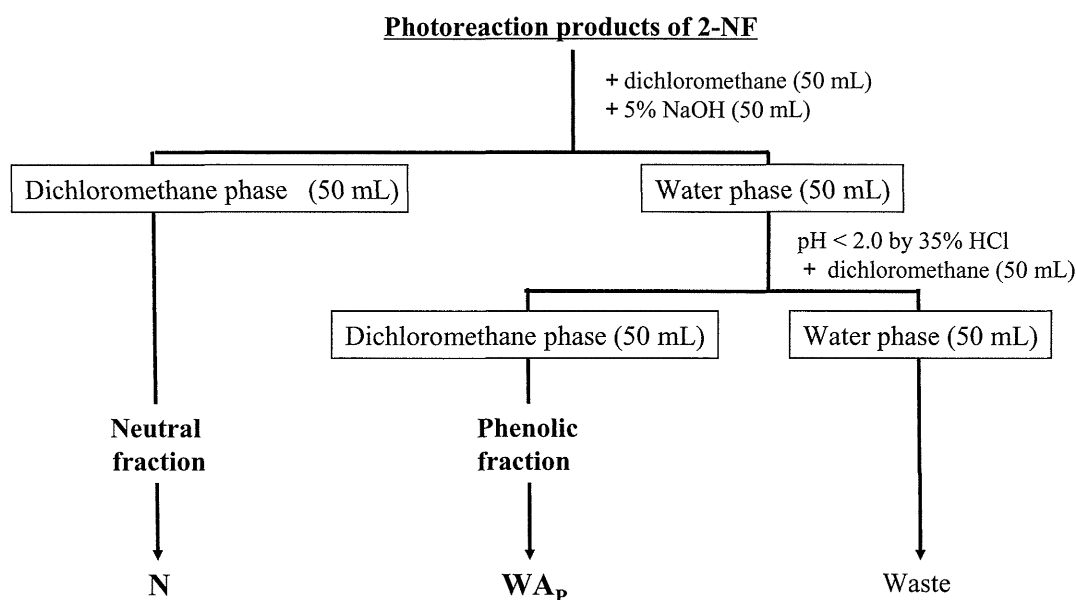

Figure 1: Fractionation of the photoreaction products of 2-NF.

equipped with a UV detector (Hitachi, L-4200, $\lambda = 254$ nm). Fractions corresponding to the OHNF isomer eluted isocratically with acetonitrile/water (1/1, v/v) at a flow rate of 10 mL min^{-1} on a Inertsil ODS-P column (GL Science, 10 mm i.d. $\times$ 250 mm) were collected separately as shown in Figure 2. The photoreaction was repeatedly carried out with ca. 10 mg (40 μmol) of 2-NF in total amount.

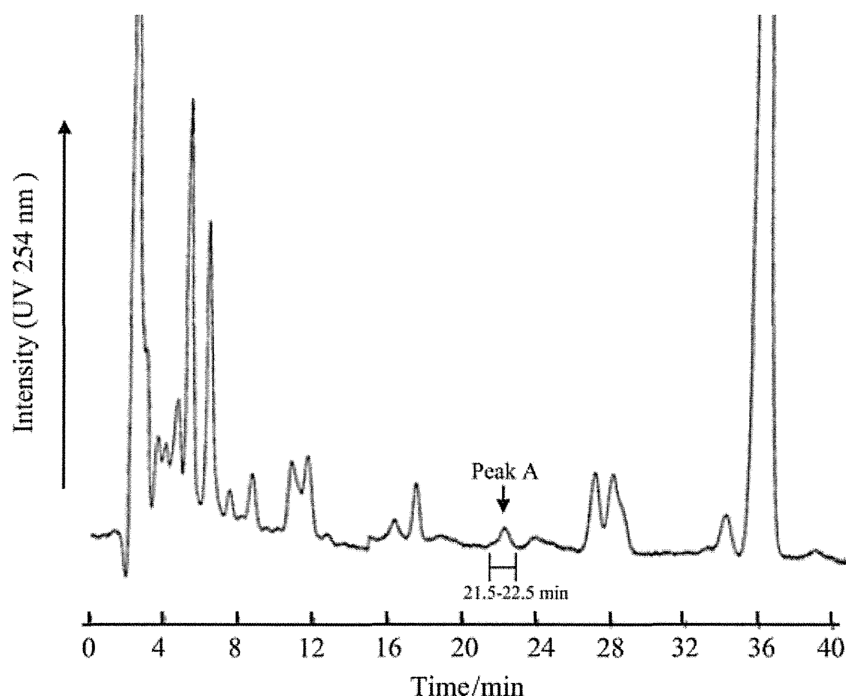

Figure 2: Chromatogram at 254 nm for HPLC fractionation of 2-NF photoreaction products using a UV detection system. A fraction from 21.5–22.5 min was collected (Peak A).

Analytical Instrumentation

LC/MS/MS analysis was performed as previously described (6) using the Agilent 1100 series LC system (Agilent Technologies) with an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems) equipped with an electrospray ionization (ESI) interface and operated in a negative ion mode. Chromatographic separation of 2-NF photoreaction products was performed on a Zorbax Extend-C18 column (150 mm × 2.1 mm i.d., Agilent Technologies). The column temperature was kept at 303 K. A gradient elution using 0.01% NH_4OH in water (eluent A) and 0.01% NH_4OH in methanol (eluent B) was carried out (B, 25–75% linear gradient for 40 min) at a flow rate of 0.2 mL min^{-1}. Sample volumes of 5 μL were typically used for each analysis. The mass spectrometer was operated under multiple reaction monitoring (MRM) mode, and the monitored precursor (Q1) and product (Q3) ions were *m/z* 262 and 232, respectively. The structures of 2-NF photoreaction products were elucidated using the enhanced product ion (EPI) scan mode in which the product ions are trapped in Q3 (in trap mode) before mass analysis. The EPI scan rate was 1,000 amu s^{-1}, and the scan range was 100–400 amu.

An HPLC system with column-switching and chemiluminescence detection previously described (6, 29) was employed for quantification of the 2-NF photoreaction product and the ambient sample. Briefly, the system consists of four HPLC pumps, a 6-port switching valve, a clean up column (GL Sciences, Inertsil ODS-P, 3.0 mm ID × 250 mm), separation columns (GL Sciences, Inertsil ODS-EP, 3.0 mm ID × 250 mm or Inertsil ODS-3, 3.0 mm ID × 250 mm × 2), a reducer column (Jasco, NPpak-RS, 4.6 mm ID × 10 mm), a trapping column (GL Sciences, Inertsil ODS-3, 4.0 mm ID × 30 mm), and a chemiluminescence detector (Soma Optics, S-3400). The chemiluminescence reagent solution was an acetonitrile solution containing 0.03 mmol L^{-1} bis(2,4,6-trichlorophenyl)oxalate and 15 mmol L^{-1} H_2O_2. Mobile phases were methanol/water (3:1, v/v) for the clean up and reduction of OHNF, and acetonitrile/imidazole-perchloric acid buffer (45:55, v/v) for the separation. The reduction of OHNF into the corresponding amino compounds, which are strongly fluorescent, was performed at 373 K in the reducer column.

Chemicals

2-NF was obtained from Chiron AS. All solvents and other chemicals used were HPLC or analytical grades from Wako Pure Chemical Ind., Ltd.

RESULTS AND DISCUSSION

Photoreaction of 2-NF

Figure 2 shows a profile of the preparative HPLC with UV absorption for the products from photoreactions of 2-NF in acetonitrile/water (3/1, v/v). Many

chromatographic peaks were observed in the chromatogram as 2-NF photoreaction products after acid-base fractionation. The HPLC fraction collected every 5 min was then analyzed by HPLC/CL which can selectively detect nitro aromatics with high sensitivity (29). A fraction obtained from 20–25 min, which contained peak A in Figure 2, yielded one major peak around 28 min in the HPLC/CL chromatogram (Figure 3a). The compound eluted around 28 min in the HPLC/CL chromatogram was also observed in the HPLC/CL analysis of a fraction obtained from 21.5–22.5 min by the HPLC fractionation (Figure 3b), which corresponds to the peak A in Figure 2.

When a 2-NF photoreaction sample was analyzed by HPLC/CL without a reducer column, the peak of the photoreaction product was completely eliminated (Figure 3c). This indicates that the photoreaction product is a nitro aromatic compound that can be reduced into a fluorescent amino aromatic compound by the reducer column. After washing the photoreaction product with 5% NaOH/water, the peak of the product also disappeared from the chromatogram (Figure 3d). This result confirms that the peak of the photoreaction product originates from an OHNF that has nitro and phenolic hydroxyl groups in its structure.

This compound gave a characteristic MS/MS spectrum with a molecular-related ion m/z 262 and fragment ions m/z 232 and m/z 216 in EPI mode of LC/MS/MS analysis (Figure 4). The similarity between the fragmentation patterns of the 2-NF photoreaction product and OHNPs (6) indicates that the product is an isomer of OHNF. The structure of the OHNF isomer obtained by the 2-NF photoreaction could not be determined by analysis of its ^{1}H-NMR spectrum due to a low yield. A lack of material also made it impossible to prepare a standard solution of the OHNF from the purified 2-NF photoreaction product.

OHNF in Ambient Airborne Particles

HPLC/CL chromatograms of the 2-NF photoreaction product and extracted SOF from airborne particles are shown in Figures 5a and 5b, respectively. A compound, whose retention time was consistent with that of the 2-NF photoreaction product, was detected by HPLC/CL. The concentration of airborne particle-bound OHNF was estimated to be 1.7 fmol m^{-3}, assuming that the response factor of OHNF for the HPLC/CL analysis was the same as that of 1-OH-6-NP. The concentration of OHNF associated with airborne particles was comparable to that of 1-OH-3-NP, but lower than that of 1-OH-6-NP by a factor of 10 (6, 29). Little is known about the concentrations of OHNF in airborne particles. The ambient airborne concentration of hydroxynitro-PAH isomers (MW 263, mainly OHNPs and OHNFs) was reported to be about 0.01 ng m^{-3} (40 fmol m^{-3}) by Nishioka et al. (30). In their study, OHNPs appear to have significantly contributed to the total concentration of hydroxynitro-PAH isomers.

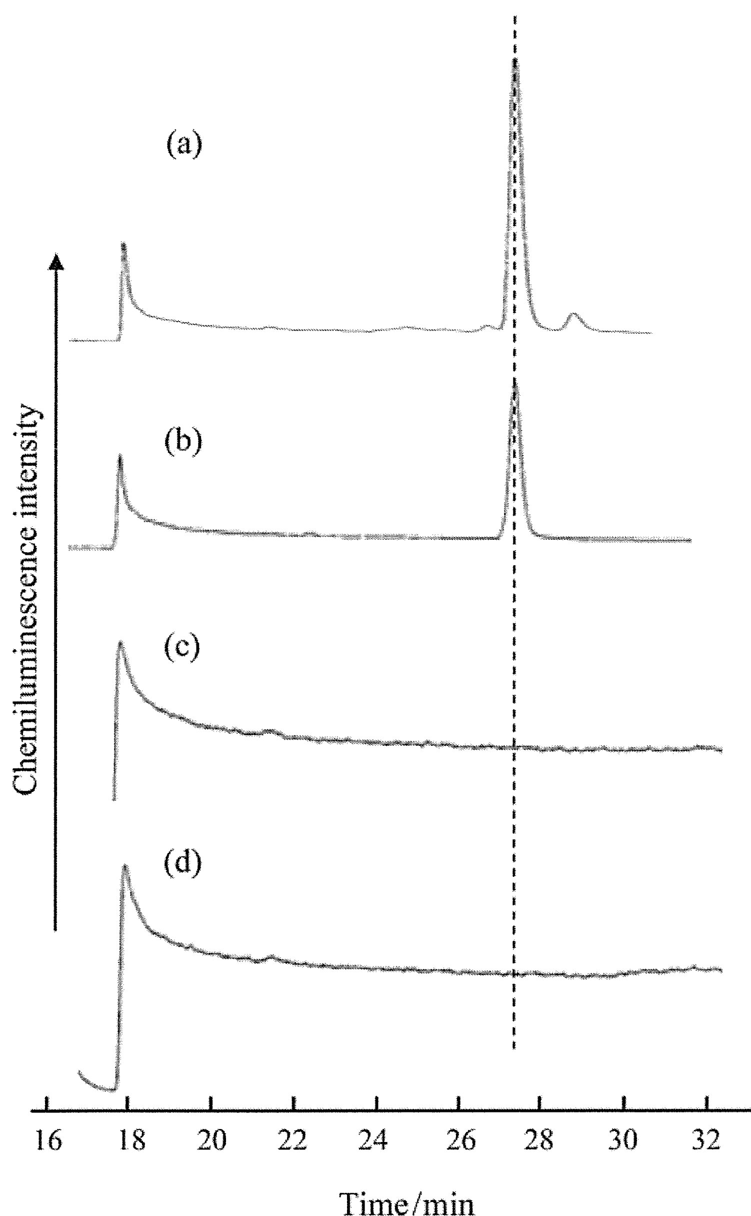

Figure 3: Typical chromatograms from the HPLC-chemiluminescence detection system for 2-NF photoreaction products after preparative HPLC fractionation: (a) fraction from 20–25 min; (b) fraction from 21.5–22.5 min; (c) fraction from 21.5–22.5 min without the reduction process; (d) fraction from 21.5–22.5 min with washing process with 5% NaOH solution. OHNF are reduced into their corresponding amino compounds in the HPLC system, and then are detected by the chemiluminescence detector. In the case without the reduction or with the washing process, the peaks of the compounds were eliminated from the chromatograms (see text for details).

Gibson et al. did not quantitatively analyze airborne particle-bound OHNF although they quantified OHNPs (5).

It has been proposed that the first step of the 1-NP photoreaction is the formation of a nitrite intermediate via both intramolecular nitro-nitrite rearrangement and C-N bond dissociation-recombination mechanisms, and that